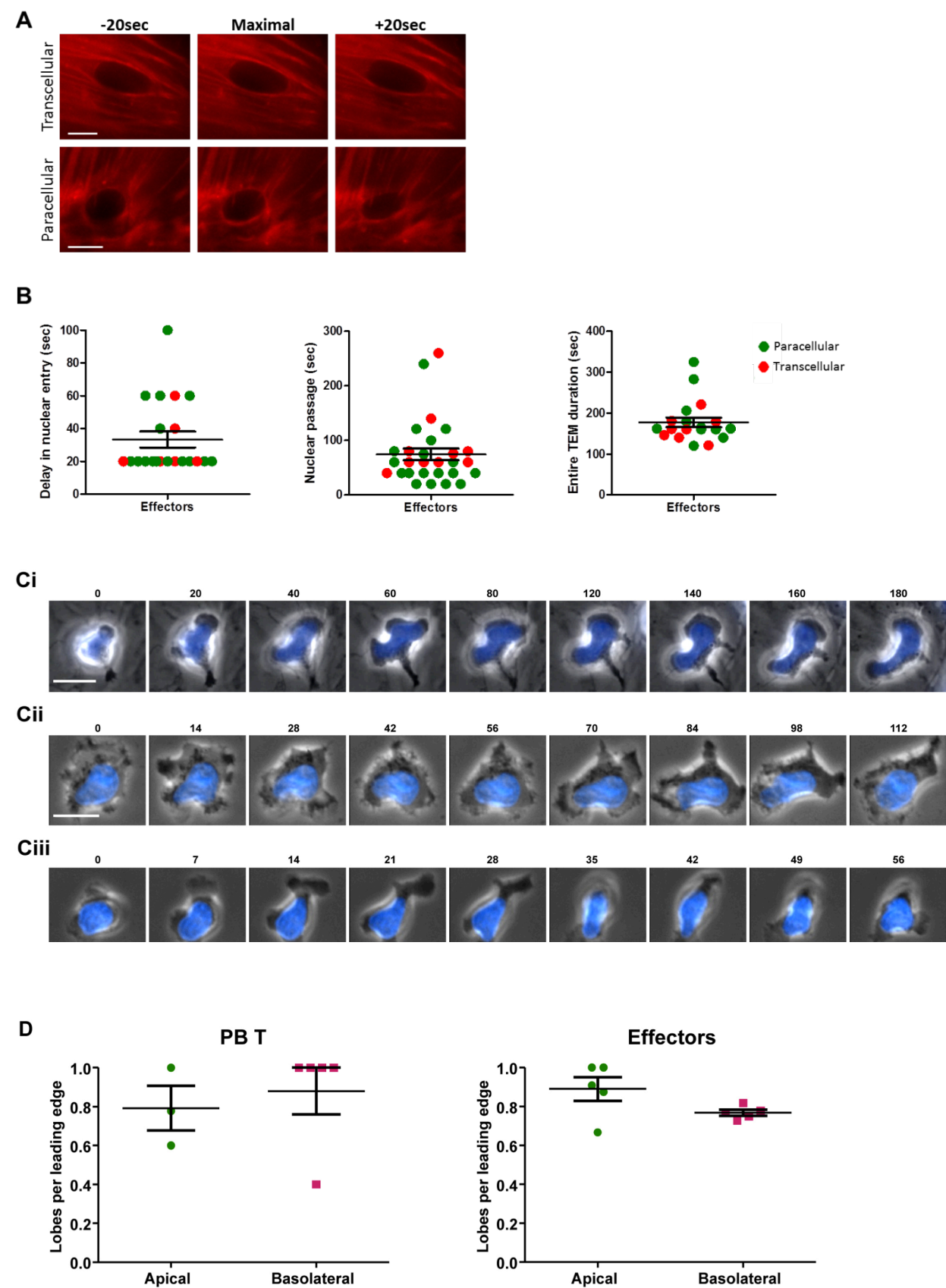


Supplemental Information

Supplemental Figures



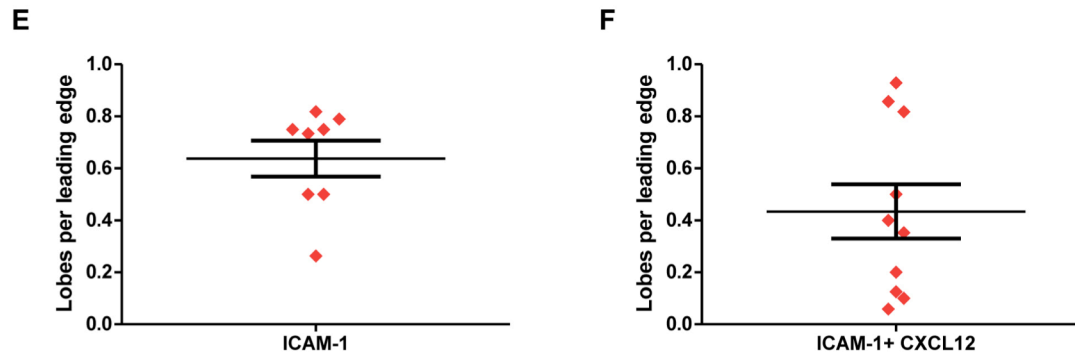


Figure S1 Related to Figure 2

Kinetics of distinct steps of paracellular and transcellular TEM of effector T cells

(A) Examples of transcellular and paracellular gaps generated during leukocyte transendothelial migration across IL-1 β stimulated HDMVECs under shear flow, as detected by the displacement of the ectopically expressed lifeact-RFP probe. Images were taken 20 sec before and after the maximal displacement by the given squeezing T cell, shown in the middle panels of each row. Scale bars= 5 μ m

(B) Kinetics of distinct steps of TEM of effector T cells through either paracellular or transcellular routes. Green and red dots denote the kinetics of the indicated steps of paracellular (green) and transcellular (red) TEM completed by individual T effector cells. **Left panel:** Delay in nuclear entry to the basolateral leading edge of individual T cells. **Middle panel:** Duration of nuclear passage of individual T cells. **Right panel:** Duration of the entire transmigration of individual T cells. (C) T cell nuclei deform and generate de novo lobes while crawling on endothelial cells or on purified ICAM-1. (Ci) Effector T cells were labeled with the nuclear dye Hoechst and allowed to crawl over HDMVEC, stimulated for 3hrs with IL-1 β . Serial images from a movie depicting a representative Hoechst labeled effector T cells during crawling under shear stress of 5 dyn/cm². Time intervals in seconds are indicated on top of each image. (Cii) Serial images of a representative Hoechst labeled effector T cell crawling on immobilized ICAM-1 at a shear stress of 5 dyn/cm². (Ciii) Serial images of a representative Hoechst labeled PB T cell crawling on ICAM-1 co-immobilized with CXCL12. T cells were allowed to interact with the substrate under shear free conditions and then subjected to a shear stress of 2 dyn/cm². Scale bars= 10 μ m. D-F Effector T cells and PB T cells generate nuclear lobes at high frequency on adhesive surfaces independently of TEM. (D) Frequency of nuclear lobe formation per leading edge generated by effector T cells or by PB T cells crawling under shear flow on (apical) or below (basolateral) IL-1 β inflamed HDMVEC monolayers. Each value represents a single Hoechst labeled cell tracked by videomicroscopy, as in C. (E) Frequency of nuclear lobe formation per leading edge generated by individual effector T cells crawling under shear flow on ICAM-1. (F) Frequency of nuclear lobe formation per leading edge generated by individual PB T cells crawling under shear flow on ICAM-1/CXCL12 substrates. The experiments analyzed in E and F were conducted as described in (C).

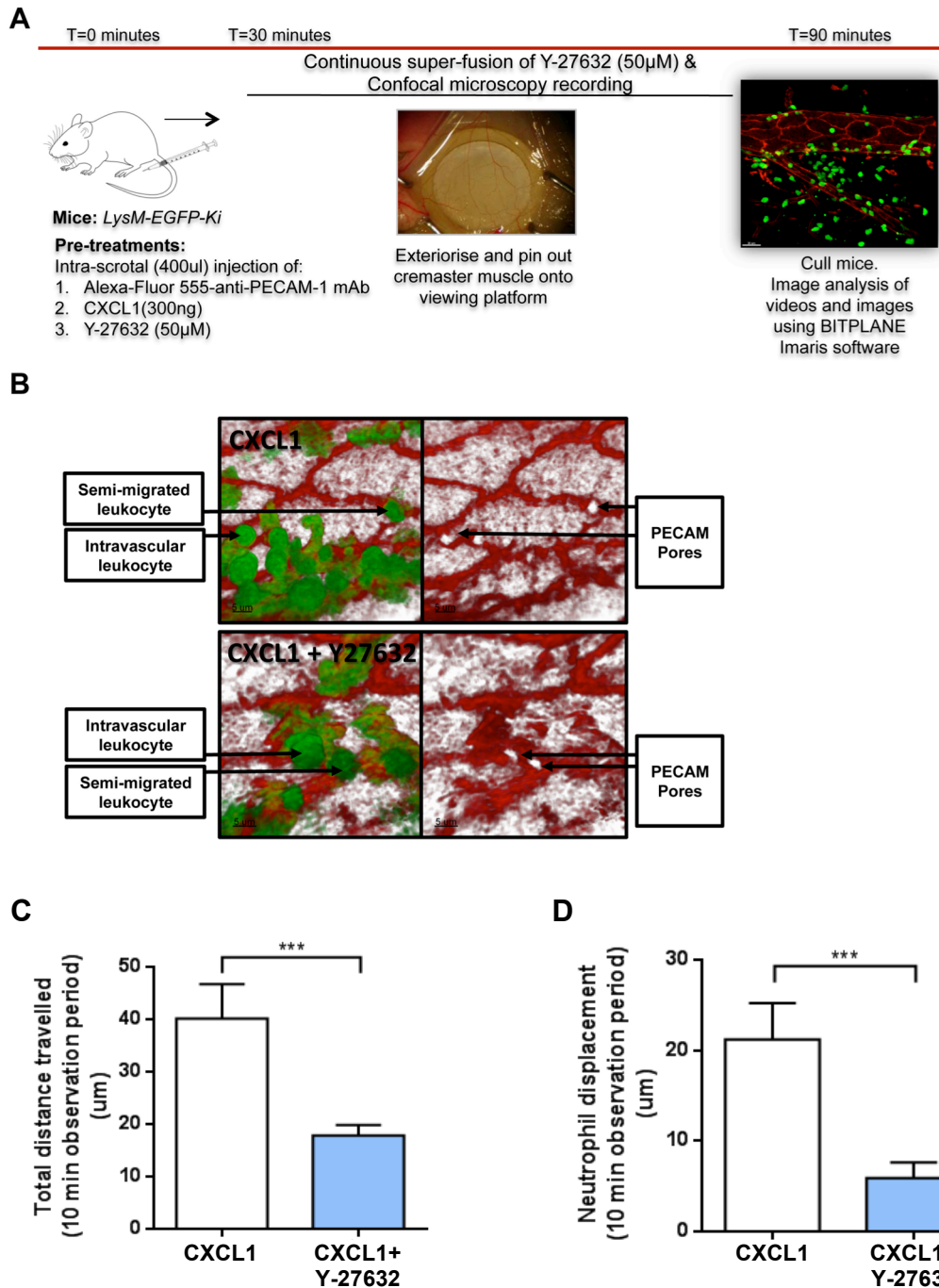


Figure S2. Related to Figure 3

Intravital microscopy protocol for testing the in vivo effects of Rho kinase inhibition on neutrophil diapedesis and interstitial motility in cremaster muscles

(A) Top line: the time course of the intra-scrotal injection of the anti PECAM-1 mAb, chemokine and Rho kinase blocker (Y-27632). Images depict the viewing platform and time lapse images recorded by confocal microscopy. For more details, see Suppl. Experimental Procedures. (B) *LysM-EGFP-Ki* mice were anaesthetized with i.p. Ketamine (125mg/kg) and Xylazine (12.5mg/kg) and maintained with Ketamine/Xylazine via intra-muscular dosing during IVM. KC (300ng) +/- Y-27632 (50 μ M final concentration) + Alexa-fluor-555-labelled anti-PECAM-1 mAb were injected via the intrascrotal route (in a total volume of 400 μ l). After 30 minutes, the cremaster muscle was surgically exteriorized for analysis by IVM as previously detailed (Woodfin et al., 2011). (C,D) Effects of intra-muscular injection of the Rho kinase inhibitor on interstitial motility of extravasating neutrophils. Neutrophil motility was analyzed by intravital microscopy of cremaster muscles perfused continuously throughout the entire course of the intravital microscopy experiment with either Y-27632 or a carrier solution, as described in Fig. S2C. (C) Total distance (in microns) traveled by endogenous EGFP labeled neutrophils outside of blood vessels. *** $P < 0.0001$. (D) Net displacement of extravasating neutrophils in microns. *** $P < 0.003$. Results in C and D are the mean values +SEM of 16 neutrophils (without Rho inhibitor), and 24 neutrophils analyzed in the presence of the Rho kinase inhibitor.

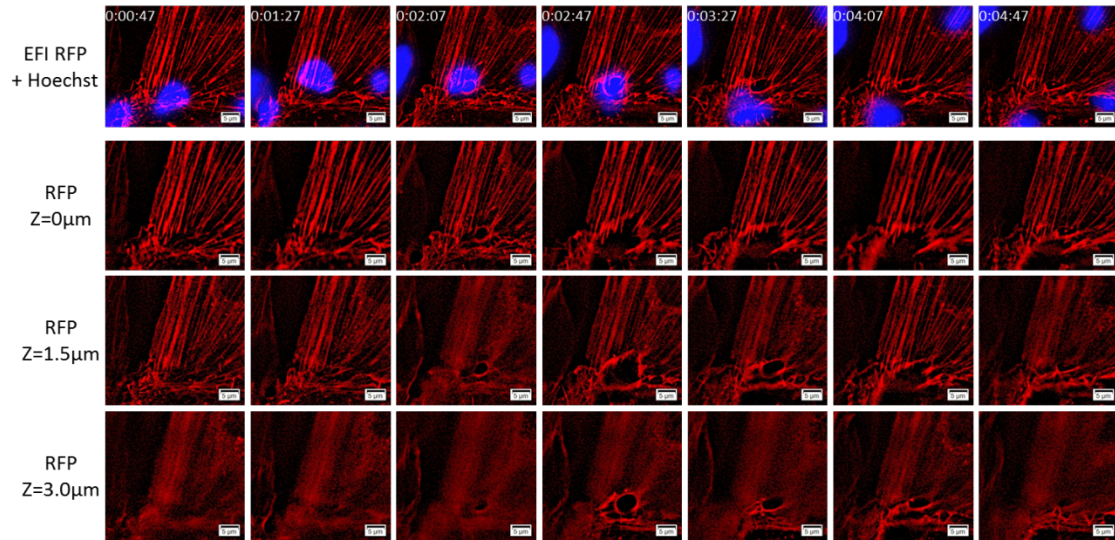


Figure S3. Related to Figure 4

Endothelial cell actin stress fibers are not disrupted during tranendothelial migration

An image panel of movie S12 showing lifting of endothelial cell stress fibers by an effector T cell that has successfully crossed the endothelial monolayer and continues to crawl below the endothelial monolayer. The top row shows reconstituted images (extended focal imaging, EFI) obtained from the three series of RFP images depicted in the bottom 3 rows. Each row corresponds to images taken at the indicated focal planes at the corresponding time points depicted in the top row. The locations of each row in the Z direction i.e., Z=0μm, Z=1.5μm and Z=3.0μm are depicted. Note the dramatic displacement of actin stress fibers by the squeezing nucleus of the T cell (starting at t=0:02:47).

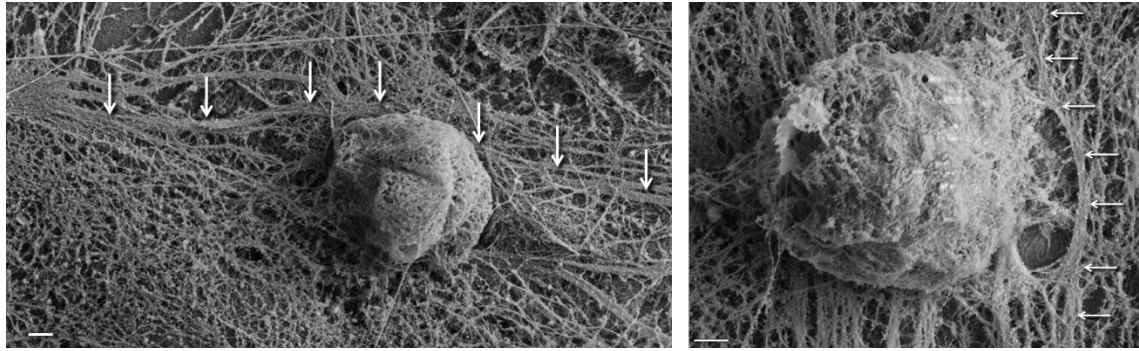


Figure S4. Related to Figure 5

SEM images of membrane stripped T cells and endothelial cells fixed during TEM

Positions of the transmigrating T cells were determined by correlative SEM videomicroscopy, as described in Figure 5. Each image depicts a representative effector T cell squeezed in between endothelial cells (left) or through cells (right) and bending and bending endothelial bundles (actin stress fibers). The bent endothelial bundles are indicated by the white arrows. Scale bars= 1 μ m.

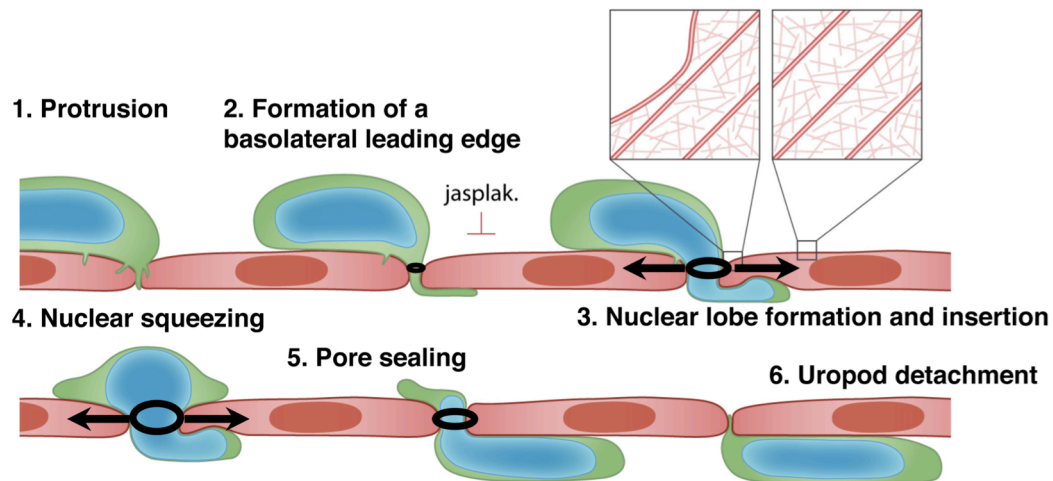


Figure S5. Related to Figures 4-7

Leukocyte TEM across flat endothelial cells consists of six main steps

A proposed model for paracellular T cell TEM based on our findings. 1. Initial actin-filled leukocyte protrusions through ECs and adherens junctions. 2. A 200-500 nm thick sub-endothelial leading edge (lamellipodium) develops underneath the EC. 3. Nuclear deformation and insertion of an anterior lobe into the sub-endothelial leading edge. This step is associated with lifting of the EC over the inserted nuclear lobe. 4. A nucleus-guided widening of the paracellular gap or transcellular pore to 4 microns. Thin endothelial actin filaments interlaced between the endothelial stress fibers undergo disassembly (not shown). 5. Sealing of the EC gap or pore by actin polymerization steps within the endothelial cells. 6. Retraction of the leukocyte uropod from the apical endothelial compartment. The TEM step most sensitive to inhibition by endothelial pretreatment with jasplakinolide (jasplak.) is shown in pink. All steps are shared by neutrophils (not shown), though they use their preexistent nuclear lobes for squeezing. Transcellular TEM of T cells follow a similar series of steps, but both initial protrusion and nuclear squeezing take place via a transcellular pore rather than through a paracellular inter-endothelial gap. The enlarged squares in step 3 depict schematic actin assemblies from imaginary top views of an endothelial cell engaged by a transmigrating leukocyte based on our Lifeact and SEM images in Figs. 4 and 5. The right square depicts endothelial stress fibers parallel to the endothelial junctions and the short actin filaments interlaced in between these fibers. The left square depicts the same stress fibers bent but not ruptured by the squeezing leukocyte nucleus. In contrast, the interlaced actin filaments are disassembled (rather than pushed aside and condensed) as a consequence of the forces exerted by the leukocyte nucleus.

Supplemental Movie Legends

All in vitro experiments of leukocyte TEM were conducted under shear flow. Unless otherwise indicated, a constant shear stress value of 5 dyn/cm^2 was used throughout the following experiments.

Movie S1 Related to Figure 2

Neutrophils rapidly insert their multilobular nuclei immediately after establishing a stable basolateral lamellipodium

Time lapse video of a representative Hoechst labeled neutrophil crossing IL-1 β -stimulated HDMVEC under shear flow. Phase contrast and fluorescence images were taken 4 sec apart. Elapsed time is designated as mm:ss. Bar, 5 μm .

Movie S2 Related to Figure 2

Effector T cells inserting multiple nuclear lobes into their multiple basolateral lamellipodia

Time lapse movie of a representative Hoechst labeled effector T cell crawling under shear flow on IL-1 β -stimulated HDMVEC. DIC and fluorescence images were taken every 6.5 sec. Elapsed time is designated as mm:ss. Bar, 5 μm . Note that the first lobe sent out by the T cell retracted immediately upon contacting the endothelial nucleus (also labeled in Hoechst, top left).

Movie S3 Related to Figure 2

Peripheral blood T cells push forward their nuclei during crawling and TEM through endothelial cells

Time lapse video of a representative Hoechst labeled PB T cell crawling and crossing IL-1 β -stimulated HDMVEC under shear flow. Transendothelial migration took place between 0:14 and 1:03 of the movie. Note the de novo lobes generated by the crawling and transmigrating lymphocyte. Phase contrast and fluorescence images were taken every 7 sec. Elapsed time is designated as mm:ss. Bar, 5 μm .

Movie S4 Related to Figure 2

Effector T cells push forward their nuclei and insert nuclear lobes into their lamellipodia during crawling under shear flow on glass coated immobilized ICAM-1 even after blocking myosin II activity

Phase contrast and fluorescence images of representative T cells allowed to adhere to the ICAM-1 coated substrate and then subjected to continuous shear flow (2 dyn/cm^2). Cells were pretreated for 30 min with the DMSO carrier (top) or with blebbistatin (bottom). Elapsed time is designated as h:mm:ss. Bar, 5 μm .

Movie S5 Related to Figure 3

Endothelial actin stress fibers of intact IL-1 β -stimulated HDMVEC are stable throughout the imaging period

A time lapse movie of a representative IL-1 β stimulated endothelial cell expressing lifeact-RFP showing steady state turnover of actin stress fiber. The DMSO carrier was added to the media at time 0:00:00 as a control. Images were taken every 1 minute. Elapsed time is designated as h:mm:ss. Bar, 20 μm .

Movie S6 Related to Figure 3

Endothelial cells actin stress fibers diminish during prolonged exposure to blebbistatin

A time lapse movie of a representative IL-1 β stimulated endothelial cell expressing lifeact-RFP showing the collapse of actin stress fibers as a function of exposure to blebbistatin. Blebbistatin was added to the media at time 0:00:00. Images were taken every 1 minute. Elapsed time is designated as h:mm:ss. Bar, 20 μm .

Movie S7 Related to Figure 2

Blocking myosin II activity in effector T cells does not inhibit nuclear deformation and lobe formation

A time lapse movie of a representative T cells prelabeled with Hoechst pretreated for 30 min with blebbistatin as in Figure 2 H-L, and allowed to adhere, crawl, and transmigrate through IL-1 β -stimulated HDMVEC under shear flow. Elapsed time is designated as mm:ss. Bar, 5 μm .

Movie S8 Related to Figure 3

Time lapse movie of IL-1 β stimulated HDMVEC treated with thrombin and recorded by phase contrast microscopy

Cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 μ m.

Movie S9 Related to Figure 3

Time lapse movie of IL-1 β stimulated HDMVEC pretreated with the Rho kinase inhibitor Y-27632 and stimulated with thrombin

The pretreated cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 μ m.

Movie S10 Related to Figure 3

Time lapse movie of IL-1 β stimulated HDMVEC pretreated with the myosin-II inhibitor blebbistatin and stimulated with thrombin

The pretreated cells were stimulated with thrombin and their contraction was imaged by phase contrast microscopy. The contours of the individual endothelial cells are outlined in different colors every 5 minutes of the experiment. Elapsed time is designated as mm:ss. Bar, 20 μ m.

Movie S11 Related to Figure 4

Transmigrating neutrophils do not rupture endothelial stress fibers during paracellular TEM

A time lapse movie depicting Hoechst labeled neutrophils crossing under shear flow through paracellular gaps of IL-1 β -stimulated HDMVEC expressing RFP-Lifeact. Note the strong Lifeact signal within the endothelial stress fibers and the actin ring formed around the squeezing neutrophils. Fluorescence images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 μ m.

Movie S12 Related to Figure 4

Transmigrating effector T cells do not rupture the endothelial stress fibers during paracellular TEM

A time lapse movie depicting Hoechst labeled effector T cell crossing under shear flow through paracellular gaps of IL-1 β -stimulated HDMVEC expressing RFP-Lifeact, a probe for filamentous actin. Note the strong Lifeact signal within the endothelial stress fibers. Phase contrast and fluorescence images were taken 20 sec apart. Elapsed time is designated as h:mm:ss. Bar, 10 μ m. For more details see also Figure S7.

Movie S13 Related to Figure 4

Transmigrating effector T cells cross inflamed endothelial cells at perijunctional sites without disrupting adjacent endothelial stress fibers

A time lapse movie depicting a Hoechst labeled effector T cell crossing through an endothelial cell near a paracellular junction of IL-1 β -stimulated HDMVEC expressing RFP-Lifeact. Note the strong Lifeact signal within the endothelial stress fibers of the left endothelial cell and their bending by the squeezing T cell. The right endothelial cell was not transfected in order to enable probing in real time the location of the edge of the two endothelial cells at their paracellular junction. DIC (green channel) and fluorescence images (T cell nucleus in blue; endothelial F-actin in red) of a time lapse movie. Images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 μ m.

Movie S14 Related to Figure 4

Stress fiber bending by the nucleus of a transmigrating effector T cell crossing transcellularly through inflamed HDMVECs

A time lapse movie depicting a Hoechst labeled effector T cell crossing through a transcellular route of IL-1 β -stimulated HDMVECs expressing RFP-Lifeact. Images were taken 20 sec apart. Elapsed time is designated as h:mm:ss. Bar, 5 μ m.

Movie S15 Related to Figure 5

Correlative videomicroscopy SEM

A time lapse movie of effector T cells crawling and crossing IL-1 β -stimulated HDMVECs under shear flow, through a paracellular route, recorded by phase contrast microscopy. Images were taken 15 sec apart. Elapsed time is designated as mm:ss. Bar, 5 μ m. At the end of this movie the sample was exposed to the lipid extraction and cytoskeleton-stabilizing solution perfused into the flow chamber as explained in the legend of Figure 5.

Movie S16 Related to Figure 7

Effect of jasplakinolide perfusion over inflamed HDMVECs on steady state actin turnover and dynamics of spontaneous endothelial ruffles

Time lapse movie showing phase contrast microscopy of an individual HDMVEC recorded shortly before and after introduction of Jasplakinolide containing medium at t=12:30. For more details, refer to the legend of Figure 7. Elapsed time is designated as mm:ss. Bar, 10 μ m.

Movie S17 Related to Figure 7

Effect of jasplakinolide perfusion over inflamed HDMVECs on actin density within endothelial ruffles

Time lapse movie showing relative actin density imaged by RFP- Lifeact. Intensity is presented in a rainbow pattern (black to red). The peripheral segment of an inflamed HDMVEC was recorded shortly before and after introduction of jasplakinolide. The time at which jasplakinolide reached the flow chamber is indicated by the appearance in the time lapse movie of the red label “Jasp”. For more details, refer to the legend of Figure 7. Elapsed time is designated as mm:ss. Bar, 10 μ m.

Movie S18 Related to Figure 7

Effect of jasplakinolide perfusion over inflamed HDMVECs on integrity of paracellular junctions.

Time lapse movie showing phase contrast images of a confluent monolayer of inflamed HDMVEC recorded shortly before and after introduction of jasplakinolide containing medium. Elapsed time is designated as mm:ss. Bar, 10 μ m.

Supplemental Experimental Procedures

Reagents and antibodies

Recombinant human CXCL12, IL-1 β and IL-2 were from PeproTech (Rocky Hill, NJ) and recombinant mouse CXCL1 was from Tocris Bioscience (Bristol, UK). Blebbistatin, bovine serum albumin (BSA), fibronectin, Hoechst 33342, polyethylene glycol, thrombin and TRITC-phalloidin, were from Sigma-Aldrich (St. Louis, MO). Jaspilakinolide and Y-27632 were purchased from Tocris Bioscience. The SiR-actin probe was purchased from Spirochrome (Stein am Rhein, Switzerland). Ca²⁺, Mg²⁺ free Hank's balanced salt solution (HBSS) was from Gibco. Anti-CD3 (OKT3), anti-CD28 (CD28.2) and anti VE-cadherin (BV9) were purchased from Biolegend (San Diego, CA).

FITC-Dextran permeability assay

Permeability across the endothelial cell (HDMVEC) monolayer was measured using 70kDa FITC-Dextran. 10⁵ HDMVECs were seeded on fibronectin-coated 24-well transwells (Costar, Corning, 3.0 μ m, Polyester membrane) and grown for 60h before the assay. Cells were stimulated with IL-1 β (2 ng/ml) for 3 and 12 hrs. 100 μ l of 1 mg/ml FITC-Dextran in HBSS containing 0.2% BSA was added to the upper chamber of the transwell and 600 μ l of FITC-Dextran free HBSS was added to the lower chamber. FITC-Dextran was allowed to diffuse from upper to the lower chamber for 60 minutes at 37⁰C. After 60 minutes, FITC-Dextran concentration in the lower chamber was measured using a fluorescence multi-well plate reader (TECAN) with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

Categories of leukocyte adhesion, crawling and transmigration analyzed in vitro

Neutrophils or T cells were perfused over the EC monolayer at 0.75 dyn/cm² for 1 min (accumulation phase) and then left under constant shear flow (5 dyn/cm²) for 10 mins. Images were video recorded through a 20x phase contrast objective at 2 frame/s. For migratory phenotype analysis, accumulated leukocytes were individually tracked throughout the assay and categorized as described in (Cinamon et al., 2001). Any leukocyte that initially accumulated but detached during the 10 min exposure to constant flow was grouped in the detachment category. Any leukocyte which arrested and remained adherent at its original site of arrest without any lateral locomotion was grouped in the arrest category. Crawling leukocytes which moved at least 1 cell diameter (~10 microns) away from their initial site of arrest but failed to cross the endothelial monolayer during the entire assay period was grouped in the crawling category. All transmigrating leukocytes (with a TEM phenotype) either crawled before crossing the endothelium or crossed the endothelium at their original point of arrest. All different groups (termed migratory phenotypes) were calculated as fractions of leukocytes which originally accumulated during the first 1 min phase.

Intravital confocal microscopy (IVM)

All experiments were carried out under UK legislation, and protocols employed were approved by the Ethical Review Committee of Queen Mary University of London. Lys-EGFP-ki mice expressing GFP-labelled myeloid cells were anaesthetized with i.p. Ketamine (125mg/kg) and Xylazine (12.5mg/kg) and maintained with Ketamine/Xylazine via i.m. dosing as previously detailed (Woodfin et al., 2011). To induce an inflammatory response, the mice were injected via the intrascrotal route (i.s.) with recombinant mouse CXCL1 (300ng in 400 μ l of PBS). Control mice received i.s. PBS. In some groups of mice, PBS and CXCL1 were co-injected with Y-27632 (50 μ M solution). All mice were additionally injected i.s. with an Alexa-555-labelled non-blocking anti-PECAM-1 mAb to stain endothelial cell junctions of the cremaster microvasculature, as previously detailed (Woodfin et al., 2011). After 30 mins, the cremaster muscle was surgically exteriorised and imaged using an upright confocal microscope (Woodfin et al., 2011). The exteriorised tissue was continuously superfused during the 1 hr observation period with warm Tyrode solution or Tyrode containing Y-27632 (50 μ M). In each mouse, 4-6 post-capillary venules were imaged in 4D, and subsequently analyzed by IMARIS Bitplane software. Neutrophil transmigration into tissues, dynamics, and the size of endothelial cell pores in the PECAM-1 channel was quantified as previously described (Colom et al., 2015; Woodfin et al., 2011).

Transient transfections in endothelial cells

HDMVEC were infected with a lentivirus (pLenti6/v5, Invitrogen Life Technologies) encoding LifeAct-RFP. Cells were harvested 1 day after infection, seeded and confluent monolayers were taken for flow experiments a day later. HDMVECs were also transfected with pEGFP- Δ N3 encoding GFP fused to the constitutively active mDIA1 mutant Δ N3 (Watanabe et al., 1999). Transfection was

performed using Amaxa Basic Nucleofector™ Kit for Primary Mammalian Endothelial Cells according to manufacturer's protocol. A day later cells were harvested and reseeded to form confluent monolayers consisting of ECs with variable levels of the GFP mDIA1 fusion protein. Leukocytes adhered and squeezed through or immediately near cells expressing high levels of the GFP fused mutant were distinguished from leukocytes interacting with identically electroporated endothelial cells lacking a detectable GFP signal.

Fluorescence microscopy

For immunostaining, samples were fixed with PBS containing 4% (wt/vol) paraformaldehyde and 2% (wt/vol) sucrose. Fixed cells were blocked with 10% (vol/vol) goat serum and incubated with either primary fluorescence-labeled mAb or unlabeled mAb, followed by secondary antibody, or with the Hoechst 33342 nuclear dye or the F-actin probe, TRITC (tetramethylrhodamine isothiocyanate)-phalloidin. Images were obtained with a PlanApo phase contrast 60× oil-immersion objective on an Olympus IX83 microscope (Olympus, Tokyo, Japan). Sections were acquired as serial z stacks and were subjected to digital deconvolution (CellSence, Olympus).

Densitometric analysis of filaments

The area of the actin fibers was determined using Fiji by a simple fixed threshold analysis and was normalized to the area of the whole image (Schindelin et al., 2012). Mean actin density was determined in at least 4 different fields of view using either 8k (1000μm²) or 25k (100μm²) magnifications.

Image analysis of nuclear shape changes

To visually illustrate the nucleus movement we prepared a 3D-stack of time lapse images of Hoechst labeled leukocytes, and created a surface rendering of the "3D-nuclei" using Imaris software. Z-spacing was 4 times the x/y spacing.

Correlative electron microscopy

Scanning electron microscopy of cellular actin networks was performed according to Hoelzle and Svitkina (Hoelzle and Svitkina, 2012). Briefly, endothelial cells and T cells were extracted in situ at 37°C for 5 min with a cytoskeleton-stabilizing solution perfused into the flow chamber (50 mM imidazole, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, and 0.5 μM TRITC-phalloidin supplemented with 1% Triton X-100 and 4% polyethylene glycol, Mr 35,000). Extracted cells were briefly washed with the same solution without Triton X-100 and polyethylene glycol, and fixed for 20 minutes with 2% glutaraldehyde in 0.1M cacodylate solution (pH=7.4) supplemented with 5mM CaCl₂ and 1% sucrose. Next, samples were fixed in aqueous solution of 0.1% Tannic acid for 20 minutes, washed twice in DDW and incubated with 0.1% uranyl acetate for an additional 20 minutes. Dehydrations were performed by washing three times with increasing ethanol concentrations (25% Vol/Vol, 50%, 75%, 100%). Prior to CPD (critical point drying) samples were incubated in 0.1% uranyl acetate in 100% ethanol for 20 minutes and washed four times in pure ethanol. After CPD the specimens were made conductive by coating with a 5 nm layer of Chromium, deposited by sputtering (Emitech K575X).

Endothelial remodeling around beads coated with anti CAM mAbs

Protein G Dynabeads (Life technologies, Carlsbad, CA) were washed 3 times in PBS and blocked with PBS containing 2% BSA (sigma) for 1h. Beads were incubated for 16 hrs at 4°C in blocking solution containing a 1:1 mixture of 0.2μg/μl anti ICAM-1 mAb (eBioscience HA58, San Diego, CA) and anti VCAM-1 mAb (Clone 4B9, a gift from Dr. T.K. Kishimoto, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). Coated beads were overlaid for 30 sec on HDMVECs monolayers prestimulated for 3 hour with IL-1β as described in the main text and let settle for 30sec. Samples were agitated for 5 minutes at 37°C and washed twice in HBSS before being exposed to the lipid extraction and cytoskeleton-stabilizing solution as described in the main text. All anti ICAM-1 and VCAM-1 antibody coated beads resisted detachment from the IL-1β stimulated HDMVEC up to a shear stress of 10 dyn/cm² whereas IgG coated Protein G beads were readily detached from the endothelial monolayer at a shear stress of 5 dyn/cm².

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